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Antibacterial activity of monoacetylated alkyl gallates against *Xanthomonas citri* subsp. *citri*

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Running title: Acetylated Alkyl Gallates target *X. citri* membrane

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Abstract

Asiatic Citrus Canker (ACC) is an incurable disease of citrus plants caused by the Gram-negative bacterium *Xanthomonas citri* subsp. *citri* (*X. citri*). It affects all the commercially important citrus varieties in the major orange producing areas around the world. Control of the pathogen requires recurrent sprays of copper formulations that accumulate in soil and water reservoirs. Here, we describe the improvement of the alkyl gallates, which are potent anti-*X. citri* compounds, intended to be used as alternatives to copper in the control of ACC. Acetylation of alkyl gallates increased their lipophilicity, which resulted in potentiation of the antibacterial activity. *X. citri* exposed to the acetylated compounds exhibited increased cell length that is consistent with the disruption of the cell division apparatus. Finally, we show that inhibition of cell division is an indirect effect that seemed to be caused by membrane permeabilization, which is apparently the primary target of the acetylated alkyl gallates.

Introduction

Xanthomonas citri subsp. *citri* is the etiological agent of Asiatic Citrus Canker, a severe disease that affects orange trees, and for which no healing process is known (Brunings and Gabriel 2003). The host range of this pathogen consists of a wide diversity of *Citrus* spp. of economic importance around the world. Symptomatic plants exhibit brownish eruptive lesions on their aerial parts, which may be surrounded by chlorotic halos. Untreated infections may lead to premature fruit-drop, stem dieback and defoliation, which is responsible for major economic losses to citriculture (Gottwald et al. 2002). *X. citri* can be introduced to new areas by the movement of infected citrus fruits and seedlings. Upon infection, the bacterium is rapidly disseminated by rainwater and wind passing over the surfaces of lesions and splashing onto uninfected nearby trees (Bock et al. 2005; Gottwald et al. 2002).

The control of citrus canker in the major orange producer area in the world, the state of São Paulo, Brazil, was satisfactorily achieved by the plant eradication program that took place between the years 1999-2009 (Belasque Jr and Behlau 2011; Belasque Jr. et al. 2009). During that period, symptomatic plants and the neighboring ones had to be eliminated to refrain the spread of the bacterium. The drawback of eradication was the high cost of visual inspections, and the enormous number of plants that had to be eliminated over the course of the years. Pressures from different sectors of the orange producing chain culminated in the current scenario in which control is exerted by the plantation of less susceptible cultivars of citrus, the use of wind-breaks to avoid bacterial lateral spreading by the combined

action of wind and rain, and the use of cupric formulations as bactericides. According to the current legislation, the state of São Paulo was declared as an area of Risk Mitigation System from 2017, and the control of citrus canker is now similar to what is already performed in the Southern states of Brazil (Behlau et al. 2008).

Concerns have now been raised about the massive use of copper as the only bactericide to control the spread of citrus canker. Copper sprays have to be applied repeatedly for effectiveness, especially after a new leaf flush, thereby control by mitigation will increase the chemical residuals left on fruits, soil, and water reservoirs. Copper can be bio-cumulative and it is a toxic metal (Brunetto et al. 2016; Cornu et al. 2017; Fones and Preston 2013). Besides, the emergence of bacterial strains resistant to copper is a fact (Behlau et al. 2012; Canteros 1999). Altogether, citriculture requires new formulations as alternatives to copper in order to combat bacterial and fungal infections.

Our research team is focused on the development of environmental friendly compounds able to combat *X. citri*. We described the use of esters of gallic acid, the alkyl gallates, as potent cell division inhibitors of *X. citri* (Król et al. 2015; Silva et al. 2013). Moreover, alkyl gallates were able to preclude the ability of *X. citri* to infect citrus plants. Finally, alkyl gallates are safer than copper, and even exhibit chemo-preventive action reducing the mutagenicity caused by agents that induce chromosomal damage (e.g. compounds that generate Reactive Oxygen Species) (Silva et al. 2017). A downside of their application in the field would be the possible broad anti-bacterial spectrum of the compounds, which may be circumvented, at least in part, by the preparation of formulations able to attach specifically to citrus leaves. In addition to this, compounds can be modified for increased potency, therefore minimizing the dose necessary for effectiveness and the need for recurrent applications in the field.

One of the strategies used to modify and perhaps improve the action of lead compounds is the optimization of physicochemical properties by the conversion of some of their functional groups. The ester group is the main alternative to the carboxyl and hydroxyl polar groups, due to the increase of lipophilicity and thus the biomembrane permeability (Beaumont et al. 2003; Rautio et al. 2008). Previous studies performed by Sardi et al. (2017) demonstrated that an acetylated derivative of curcumin, a natural polyphenolic compound, was more potent than its natural prototype against *Staphylococcus aureus* strains, showing the importance of converting hydroxyl to ester groups for antibacterial activity. Here we demonstrate that acetylation of some of the previously described alkyl gallates increased 100% their potency against *X. citri*. Compounds stimulated morphological alterations in *X. citri*, which is consistent

with a disruption of the bacterial cell division process. However, our data support the view that the action on division is indirect and a consequence of breakage of the cell transmembrane potential, which is required for the correct assembly/positioning of the divisome.

Materials and methods

Synthesis and ^{1}H NMR spectrum data of monoacetylated alkyl gallates

Monoacetylated alkyl gallates were synthesized by the acetylation of alkyl gallates according to Changtam et al. (2010) with minor modifications. First, alkyl gallates with side chains varying from five to eight carbons were synthetized as described in Silva et al. (2013). Next, acetic anhydride (10 mL) was added to the solutions containing the alkyl gallates (1 mmol) in pyridine (10 mL), mixtures were stirred at 100 °C for 7 days, and monitored by successive TLC analyses. When reactions were finished, residues were poured into crushed ice. The resulting solutions were partitioned with ethyl acetate and the organic phase dried at room temperature. The crude products were purified over silica gel column eluted with mixtures of hexane and ethyl acetate, furnishing monoacetylated alkyl gallates (**8a− 11a**). Compound numbers were chosen to keep in line with our previous reports (Krol et al. 2015; Silva et al. 2013). NMR spectra were recorded at 600 MHz for ^{1}H nucleus on a Bruker Avance III spectrometer at 25 °C.

Bacterial strains and media

The *Xanthomonas citri* subsp. *citri* used was the sequenced strain 306 (IBSBF-1594) (da Silva et al. 2002; Schaad et al. 2006). *X. citri amy*::pPM2a-*zapA*, expressing GFP-ZapA (Martins et al. 2010), was used to monitor the possible action of test compounds on the bacterial divisional septa. Cells were cultivated at 30 °C under rotation (200 rpm) in NYG/NYG agar medium (peptone 5 g/L, yeast extract 3 g/L and glycerol 20 g/L). Kanamycin and ampicillin were used at 20 µg/mL.

Compound susceptibility test

The antibacterial action of the acetylated alkyl gallates was measured by the resazurin microtiter assay (REMA) described in Silva et al. (2013). Stock solutions of compounds at 10 mg/mL were prepared by

dissolving the acetylated alkyl gallates (dried-powder samples) in 100% dimethyl sulfoxide (DMSO; SIGMA 276855). Test suspensions of acetylated alkyl gallates were prepared straight into 96-microtiter wells by diluting the stock solutions with NYG medium using a two-fold serial dilution scheme. The initial test concentration of a given compound was 100 µg/mL and 1% DMSO, and each well contained a total volume of 100 µL. Cell inoculum was prepared by diluting an overnight culture of *X. citri* in NYG medium to make a suspension at 10^7 CFU/mL. Ten microliters of this cell suspension was distributed into the wells of the above-mentioned 96-microtiter plate so to give a final inoculum concentration of 10^5 cells/well. The negative control consisted of NYG medium and the bacterial inoculum. Kanamycin (20 µg/mL) and 1% DMSO were used as positive and vehicle control, respectively. After the tests assembly, plates were incubated for 4 hours at 30 ºC. In order to develop the assay, 15 µL of a 0.01% resazurin (SIGMA R7017) were added to each well followed by a further incubation period of 2 hours at 30 °C. Viable cells were determined by their ability to reduce the blue resazurin dye to the pink fluorescent compound resorufin, which was detected using a fluorescence scanner Synergy H1MFD (BioTek), with excitation and emission wavelengths set to 530 and 590 nm, respectively. Three independent experiments were conducted, and the data were used to construct plots of chemical concentration versus cell growth inhibition in order to determine the MIC90 and MIC50 values (the concentration of a given compound able to inhibit 90% and 50% of the cells in a culture, respectively). To investigate if the acetylated alkyl gallates had bactericidal or bacteriostatic activities, we plated samples (~10 µL) of the cell suspensions exposed to the compounds in REMA just before adding resazurin. Plating was done on solid NYG medium containing ampicillin (20 µg/mL) using a 96-replica plater (8 X 12; SIGMA). Plates were incubated at 30 °C for 48h, and experiments were performed in triplicates. The bacteriostatic action was defined by the ability of a compound, at a specific concentration, to preclude bacterial respiration as measured in the REMA assay, but cells can still grow after cultured in the absence of the compound. The concentration of a given compound was considered bactericidal when bacterial growth was not observed after plating on NYG-agar.

Cell morphology and septum disruption analyses

Overnight cultures of *X. citri* and the mutant strain *X. citri amy*::pPM2a-*zapA* were diluted 1:100 into fresh NYG medium and cultivated at 30 ºC and 200 rpm until the OD600nm of ~ 0.7. One milliliter of culture was treated with the compounds at MIC50 or 1% DMSO for 6 hours at 30 ºC. Cells were

immobilized on 1% agarose (0.9% NaCl)-covered slides and observed using a fluorescence microscope BX-61 (Olympus) equipped with a monochromatic camera OrcaFlash 2.8 (Hamamatsu). Image documentation and processing were conducted using the software Cell-Sens version 11 (Olympus).

Membrane permeability assay

Overnight cultures of *X. citri* were diluted 1:100 into fresh NYG medium and cultivated at 30 ºC and 200 rpm until the OD600nm of ~0.7. Approximately 1 mL of cell suspension was exposed to the compounds at MIC50 or the vehicle control 1% DMSO for 60 minutes at 30 ºC. A positive control for membrane permeability was performed using heat shock at 55 ºC for 2 min. Cell samples were concentrated by centrifugation for 30 seconds at 11.000 x g and the pellets were dissolved in 70 µL of 0.9% NaCl. The membrane integrity was assessed using the Live/Dead BacLight bacterial viability kit (Invitrogen) according to the manufacturer's instructions. After treatment, cells were concentrated by centrifugation, and the pellets were dissolved in 1 mL of 0.9 % NaCl prior to microscope observation.

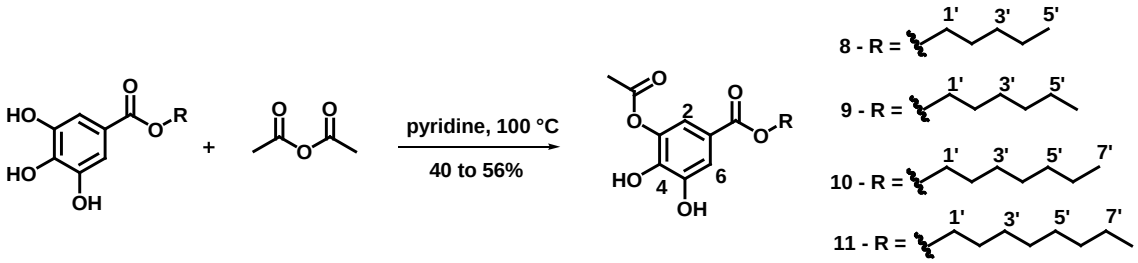
Data analyses

Dose-response curves were generated using data from three independent REMA experiments. The minimal inhibitory concentration (MIC) values were determined using the regression curves generated by the best-fit method available in the software package GraphPad-Prism 6. Statistical analyses of cell length were performed using one-way analysis of variance (ANOVA) followed by a Tukey posttest ($P < 0.05$).

Results

Synthesis and ^{1}H NMR spectrum data of monoacetylated alkyl gallates

The monoacetylated alkyl gallates carrying the alkyl radicals pentyl, hexyl, heptyl and octyl (compounds **8a**, **9a**, **10a** and **11a**, respectively) were synthesized with yields ranging from 40 to 56 % (Scheme 1).



Scheme 1. Synthesis of monoacetylated alkyl gallates (**8a−11a**)

The signals that certify the achievements of **8a−11a** correspond to the singlet in 2.4 ppm, relative to the hydrogens of the acetyl group and two doublets relating to hydrogens H-2 and H-6, which indicate loss of chemical equivalence due to the insertion of the acetyl group. For all compounds, NMR parameters corresponded with the proposed structures.

Monoacetylated pentyl gallate (8a): pentyl 3-acetoxy-4,5-dihydroxybenzoate. White solid. 40 % yield. ^{1}H NMR (600 MHz; $CDCl_3$) δ_H (mult.; *J* in Hz): 7.55 (d; 1.8; H-2), 7.39 (d; 1.8; H-6), 4,29 (t; 6.0; H-1'), 2.40 (s; 3-OCOC**H**$_3$), 1.74 (m; H-2'), 1.41 to 1.30 (m; H-3' and H-4'), 0.90 (t; 7.2; H-5').

Monoacetylated hexyl gallate (9a): hexyl 3-acetoxy-4,5-dihydroxybenzoate. White solid. 53 % yield. ^{1}H NMR (600 MHz; $CDCl_3$) δ_H (mult.; *J* in Hz): 7.52 (d; 2.4; H-2), 7.40 (d; 2.4; H-6), 4.29 (t; 6.6; H-1'), 2.41 (s; 3-OCOC**H**$_3$), 1.75 (m; H-2'), 1.44 to 1.32 (m, H-3' to H-5'), 0.92 (t; 7.2; H-7').

Monoacetylated hepyl gallate (10a): heptyl 3-acetoxy-4,5-dihydroxybenzoate. White solid. 49 % yield. ^{1}H NMR (600 MHz; $CDCl_3$) δ_H (mult.; *J* in Hz): 7.50 (d; 1.8; H-2), 7.38 (d; 1.8; H-6), 4.28 (t; 6.6; H-1'), 2.40 (s; 3-OCOC**H**$_3$), 1.76 (m; H-2'), 1.44 to 1.30 (m; H-3' to H-6'), 0.91 (t; 6.6; H-7').

Monoacetylated octyl gallate (11a): octyl 3-acetoxy-4,5-dihydroxybenzoate. White solid. 56 % yield. ^{1}H NMR (600 MHz; $CDCl_3$) δ_H (mult.; *J* in Hz): 7.52 (d; 1.8; H-2), 7.40 (d; 1.8; H-6), 4.29 (t; 6.6; H-1'), 2.41 (s; 3-OCOC**H**$_3$), 1.76 (m; H-2'), 1.44 to 1.31 (m; H-3' to H-7'), 0.91 (t; 7.2; H-8').

Acetylated alkyl gallates inhibit growth of *X. citri*

The antibacterial potential of the acetylated alkyl gallates was evaluated using REMA, a method that allows the measurement of the bacterial cell respiratory activity. All of the compounds tested exhibited strong inhibition of *X. citri* with minimum inhibitory concentration (MIC) values ranging from

187 approximately 28 to 46 µg/mL, which are nearer to the value of the positive control kanamycin (20
188 µg/mL) (Table 1). The anti-*X. citri* activity of the monoacetylated alkyl gallates (**8a−11a**) indicated a
189 correlation among MIC values and the length of alkyl side chains. Note that the MIC values decreased
190 with the increase of the alkyl chain: pentyl (MIC 45.73 µg/mL) < hexyl (MIC 34.65 µg/mL) < heptyl
191 (MIC 31.97 µg/mL) < octyl (MIC 27.92 µg/mL). The same correlation was observed in the activity of the
192 non-acetylated alkyl gallates (**8−11**) (Silva et al. 2013). However, when we compare the MIC values of
193 the non-acetylated alkyl gallates (**8, 9, 10** and **11** exhibited MICs of ~60 µg/mL) with the acetylated alkyl
194 gallates (~28 - 46 µg/mL) we detect a clear increase of potency. Here, the antibacterial activities of the
195 acetylated compounds increased 36% for **8a**, 80% for **9a**, 95% for **10a**, and 123% for **11a** (Table 1).

196 As lipophilicity is a central parameter for the development of novel bioactive compounds we
197 determined the theoretical lipophilicity (C log P) of the acetylated alkyl gallates (Table 1). As expected,
198 the acetylation of alkyl gallates led to an increase of lipophilicity in the order of ~22%. The non-
199 acetylated alkyl gallates **8-11** (Silva et al. 2013) displayed C log P values ranging from 2.53 to 3.72, while
200 the acetylated forms **8a-11a** started with a C log P value of 3.12 and ended in 4.51 (Table 1). Taken
201 together, our results indicate that the increase in lipophilicity induced by the esterification of hydroxyl
202 groups resulted in increased potency of the acetylated derivatives against *X. citri*.

203 Finally, we determined the minimal bactericidal concentration (MBC) of the acetylated alkyl
204 gallates. The MBC is defined as the lowest concentration capable of inhibiting growth of 99.99% of the
205 bacterial inoculum (NCCLS 2003). *X. citri* was exposed to a concentration range of the compounds
206 varying from 12.5 to 100 µg/mL following the same procedure used in REMA. After treatment, cell
207 suspensions were plated on NYG-agar and incubated for up to 48h to score for colony development. In
208 our evaluation, compound **8a** had only bacteriostatic action, where the highest dose used (100 µg/mL)
209 was not enough to prevent cell growth on plate (Table 2). Note that the dose of 100 µg/mL is twice as
210 much as the dose that led to a growth halt in REMA (45.73 µg/mL; Table 1). For the three remaining
211 compounds (**9a**, **10a**, and **11a**), the bactericidal dose was related to the size of carbon side chain (Table
212 2). Compound **9a,** with the shortest carbon side chain of the three, exhibited a MBC between 50-100
213 µg/mL (the exact concentration was not determined); the concentration of 50 µg/mL for compound **9a**
214 was therefore considered bacteriostatic. Compounds **10a** and **11a** displayed MBC values in the ranges of
215 25-50 µg/mL and 12.5-25 µg/mL, respectively, with the concentrations of 25 µg/mL and 12.5 µg/mL
216 being considered bacteriostatic (Table 2).

Acetylated alkyl gallates induce morphological changes in *X. citri*

In our previous work with the non-acetylated versions of the alkyl gallates, we showed that these compounds induce filamentation in *B. subtilis* and increased cell size in *X. citri,* which may reflect interference with the bacterial cell division process (Król et al. 2015; Silva et al. 2013). To investigate if the acetylated alkyl gallates had the same mechanism of action, we examined the morphology of *X. citri* exposed to these compounds. Wild-type cells of *X. citri* were exposed to the compounds for 6 hours, and after analyzed under the microscope. First, the average cell length determined for the untreated cells was 1.7 ± 0.36 μm (Table 3). Treatment with compounds **10a** and **11a** led to a significant increase of cell size, which now reached 1.94 ± 0.39 and 2.07 ± 0.40 μm, respectively. The average cell length in cultures exposed to the compounds **8a** and **9a** did not differ significantly from the control (untreated). Overall, the acetylated alkyl gallates of longer carbon side chains (compounds **10a** and **11a**) induced morphological alterations in *X. citri.*

Septum disruption in *X. citri*

The cell elongation phenotype induced in *B. subtilis* and *X. citri* by the alkyl gallates **8-11** was in part explained by the direct interaction of these compounds with the cell division protein FtsZ (Król et al. 2015; Silva et al. 2013). In order to evaluate if the acetylated derivatives could target the divisome as well, we monitored the integrity of the divisional septum of *X. citri* treated with the compounds. This was possible by the use of a *X. citri* mutant strain (*X. citri amy*::pPM2a-*zapA*; (Martins et al. 2010)) expressing the FtsZ accessory protein ZapA, as GFP-ZapA, which labels the Z-ring. A normal septum can be observed in dividing cells of *X. citri amy*::pPM2a-*zapA* as a fluorescent bar perpendicular to the long axis of the rod (Fig. 1A; white arrow). Treatment with the vehicle DMSO did not interfere with the Z-ring (Fig. 1B). However, the exposure of the cells to the compounds **10a** and **11a** for 10 min at MIC50 dissolved the septa and the GFP-ZapA fluorescence is now scattered within the rods (Fig. 1C, 1D). The compounds **8a** and **9a** had no noticeable effect on the septa, which displayed a normal microscopic pattern (data not shown). Taken together, results indicate that the acetylation of compounds **10a** and **11a** kept their ability to disrupt the Z-ring of *X. citri*; however, the acetylation of compounds **8a** and **9a**

apparently abolished this property that was observed before in *B. subtilis* and *X. citri* (Król et al. 2015; Silva et al. 2013).

Membrane integrity is affected by acetylated alkyl gallates

After observing that compounds **10a** and **11a** could disrupt the divisional septum of *X. citri*, we wondered if they were capable of targeting FtsZ directly. To evaluate for that we monitored if compounds **10a** and **11a** could interfere with the polymerization/associated GTPase activity of FtsZ. Purified *B. subtilis* FtsZ was combined with various concentrations of the compounds in a pre-polymerization buffer (without nucleotides), and the reaction was initiated by adding 1 mM GTP. The GTP hydrolysis rate was determined by the generation of Pi as described in Król et al. (2015). Surprisingly, we did not observe any effect on the GTPase activity of purified *B. subtilis* FtsZ in the presence of compounds **10a** and **11a** (data not shown), which raised the possibility that the compounds may perturb the divisome indirectly.

It has been demonstrated that disruption of the membrane potential interferes with the localization of proteins like FtsZ (Strahl and Hamoen 2010). Moreover, we reported recently that alkyl gallates **10** and **11** target both purified FtsZ and the bacterial membrane of *B. subtilis* (Król et al. 2015). To verify if the acetylated alkyl gallates **10a** and **11a** could produce similar effects, we monitored the *X. citri* membrane integrity using the nucleic acid dyes SYTO 9 and Propidium Iodide (PI). PI penetrates the cells with damaged membranes as can be seen after a heat shock treatment (HS; Fig. 2). Healthy membranes are not permeable to PI, and in general, normally growing *X. citri* will have ~5% of cells with compromised membranes (NC; Fig. 2). Exposure to the vehicle DMSO at 1% did not alter this pattern. However, treatment with the four acetylated compounds **8a-11a** at MIC50 led to significant increases of permeability (Fig. 2). Noteworthy, the extent of membrane damage seems correlated to either the lipophilicity or the size of the carbon chain of these compounds. The longer the side chain the worse the effect on *X. citri* membrane. Taken together, data support that the acetylated alkyl gallates may act indirectly on the divisome via disruption of membrane integrity.

Discussion

Esters of gallic acid (the alkyl gallates) are potent growth inhibitors of *X. citri* and *Bacillus subtilis*, displaying as mechanism of action a combined activity against the divisome and the bacterial membrane (Król et al. 2015; Silva et al. 2013). Upon treatment with these compounds *X. citri* lost the ability to colonize the host citrus and to produce disease symptoms (Silva et al. 2013). Here, we designed and synthesized four alkyl gallate derivatives (the acetylated alkyl gallates **8a− 11a**) identified as new chemical entities, which also inhibited the phytopathogen *X. citri*, but exhibited greater potency than their prototypes. Our data are in line with reports from other groups showing that acetylated derivatives can improve potency when compared to their starting compounds (Biasutto et al. 2007; Sardi et al. 2017; Vlachogianni et al. 2015). The conversion of functional groups, known as drug latentiation, results in increased lipophilicity that may be related to greater capacity of penetration into biomembranes (Ettmayer et al. 2004; Han and Amidon 2000). One of the reasons why we chose to design the monoacetylated alkyl gallates instead of di- or tri-acetylated derivatives is associated to the appropriate balance between lipophilicity and hydrophilicity. This balance ensures high permeability through biological membranes and solubility in aqueous medium, which are determinant factors for the success of bioactive compounds (Dahan et al. 2016; Lipinski 2000). In addition, compounds with higher lipophilicity are correlated to the high environmental toxicity of some pesticides, which is due to their tendency to be accumulated in plants and animals (Zhang et al. 2016). Finally, the immediate advantage of the acetylated alkyl gallates would be the lower effective dose to be used for bacterial control and a lower environmental impact in agriculture. We are developing formulations containing gallates as alternatives to copper that is the bactericide currently in use to refrain the spread of the bacterium in the orchards (Behlau et al. 2010).

Copper formulations have long been utilized in citriculture, as well as in other cultures, to protect them against bacterial and fungal infections (Fones and Preston 2013; Leite Jr and Mohan 1990). Despite its efficacy, several environmental toxicity problems can be associated to the excessive use of copper as a crop defensive, which soon may call for a ban of its use worldwide (reviewed by Fones and Preston in 2013). It is worth mentioning, it was reported that copper induces viable but nonculturable state (VBNC) in *X. citri*, which consequently will lead to reduced protection irrespective of the dose and frequency of copper application (del Campo et al. 2009). Another outcome of the long-term exposure to this metal is the already documented emergence of copper-resistant strains of *X. citri* (Behlau et al. 2012; Canteros 1999). So far, we were not able to isolate strains of *X. citri* resistant to the gallates in laboratory

controlled culture (data not shown), and this may be related to the fact that alkyl gallates have a suggested multi-target mechanism of action (Król et al. 2015). Finally, the non-acetylated alkyl gallates were evaluated in a set of *in vitro* experiments as non-cytotoxic, and non-genotoxic/mutagenic compounds; moreover, they exhibit a desirable chemopreventive action being able to protect cells against chemically induced chromosomal damage (Silva et al. 2017). These observations make the gallates a safer alternative to copper to be adapted in citriculture.

The reported action of the gallates on the bacterial divisome was attributed to the inhibition of the FtsZ function (Król et al. 2015). FtsZ is the bacterial tubulin that assembles into protofilaments and organizes a ring-like structure (the Z-ring; divisional septum) in the middle of the cells to orchestrate the recruitment of all the proteins necessary for cytokinesis and cell wall remodeling/synthesis (reviewed by Erickson et al. in 2010). Conservation within the domain Bacteria and a rather dissimilarity with eukaryotic tubulins make of FtsZ an interesting target for antimicrobials. Several of the compounds that target FtsZ do so by inhibiting its GTPase activity, which consequently over-stabilizes the FtsZ protofilaments and break its assembly/disassembly dynamics needed for proper cell division function (Hurley et al. 2016). We showed previously that alkyl gallates **8-11** inhibited the GTPase activity of *B. subtilis* FtsZ (Król et al. 2015). Although, strong binding to FtsZ was observed only for compounds **10** and **11** (Kd values of 0.08 and 0.84 μM, respectively), while binding of **8** and **9** seems aspecific. Consistent with our previous data, the derivatives **10a** and **11a** induced morphological alterations in *X. citri*, documented as increased cell length, as well as disruption of the divisional septa (Fig. 1C, 1D). However, **10a** and **11a** lost the ability to interact with FtsZ, since these compounds no longer inhibited the GTPase activity of purified *B. subtilis* FtsZ. One possibility raised to explain how **10a** and **11a** dissolved the bacterial septum was because they kept their ability to act on membranes (Fig. 2) (Król et al. 2015). The increased lipophilicity of **10a** and **11a** may explain, in part, the higher potency if compared to the prototypes, and their ability and/or preference to attack the bacterial membrane. Disruption of the cell transmembrane potential, e.g. by membrane permeabilization, interferes with the localization of protein factors necessary for the Z-ring mid-cell assembly (Strahl and Hamoen 2010). Although **8a** and **9a** did not alter cell morphology and septum assembly, they kept the capacity to act on membranes, which probably confer to these compounds a marginal/undetectable effect. Therefore, our data suggest that the increase of lipophilicity in monoacetyl derivatives of alkyl gallates enhanced their anti-*X. citri* activity while

maintaining their ability to act on membranes. However, the conversion of a hydroxyl to an acetyl group resulted in loss of the ability to interact with FtsZ.

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Figures legends

Fig. 1. Acetylated alkyl gallates disrupt the divisional septum of *X. citri*. The mutant strain *X. citri amy*::pPM2a-*zapA*, expressing GFP-ZapA, was cultivated until the O.D.600nm of ~0.3, and then subjected to the acetylated alkyl gallates at MIC50 for 10 min prior to microscope observation. A) Untreated; B) cells exposed to 1% DMSO; C) **10a**, and D) **11a**. The divisional septum is marked with white arrows in A and B. DIC: Differential Interference Contrast microscopy. Scale bars correspond to 2μm; magnification 100X.

Fig. 2. Membrane integrity is affected by the acetylated alkyl gallates. Cells of *X. citri* were incubated for 1h with 1% DMSO and the compounds at MIC50. Following this period, membrane integrity was assessed using the Live/Dead kit. NC, untreated; HS, Heat Shock for 2 minutes at 55 ºC (positive control for membrane permeabilization); **8a, 9a, 10a**, and **11a** compounds as in Table 1. This experiment was performed twice with n=250 of cells scored per treatment. Bars represent the average values for the combined experiments/treatments; vertical lines indicate the standard deviation.

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